A novel pink-pigmented facultative *Methylobacterium phyllosphaerae* sp. nov. from phyllosphere of rice

Ki-Yoon Kim, M. Madhaiyan, Woo-Jong Yim, P.S. Chauhan and Tong-Min Sa*

Department of Agricultural Chemistry, Chungbuk National University, Cheongju, Chungbuk, 361-763, Republic of Korea.
E-mail: tomsa@chungbuk.ac.kr

Abstract

A pink-pigmented, aerobic, facultatively methylotrophic bacterial strain, CBMB27^T, isolated from leaf tissues of rice (*Oryza sativa* L.), was analysed using a polyphasic taxonomic approach. Comparative 16S rRNA gene sequence-based phylogenetic analysis placed the strain in a clade with the species *Methylobacterium oryzae, Methylobacterium fujisawaense* and *Methylobacterium mesophilicum*; strain CBMB27^T showed sequence similarities of 98.3, 98.5 and 97.3 %, respectively, to the type strains of these three species. DNA–DNA hybridization experiments revealed low levels of DNA–DNA relatedness between strain CBMB27^T and its closest relatives. The sequence of the 1-aminocyclopropane-1-carboxylate deaminase gene (*acdS*) in strain CBMB27^T differed from those of close relatives. The major fatty acid of the isolate was $C_{18:1}$ ω7c and the G+C content of the genomic DNA was 66.8 mol%. Based on the results of 16S rRNA gene sequence analysis, DNA–DNA hybridization, and physiological and biochemical characterization, which enabled the isolate to be differentiated from all recognized species of the genus *Methylobacterium*, it was concluded that strain CBMB27^T represents a novel species in the genus *Methylobacterium* for which the name *Methylobacterium phyllosphaerae* sp. nov. is proposed (type strain CBMB27^T =LMG 24361^T =KACC 11716^T =DSM 19779^T).

Key Words

ACC, 1-aminocyclopropane-1-carboxylate, PPFM, pinkpigmented, facultatively methylotrophic.

Introduction

Bacteria of the genus *Methylobacterium*, class *Alphaproteobacteria*, consist mainly of a group of pinkpigmented facultatively methylotrophic bacteria with the ability to utilize C1 compounds such as methanol or formaldehyde and other, multicarbon compounds (Green 1992). Cells are strictly aerobic, Gram-negative rods and, at the time of writing, the genus *Methylobacterium* comprised 28 species with validly published names, with *Methylobacterium organophilum* as the type species (Patt *et al.* 1976). Members of the genus *Methylobacterium* are versatile in nature and ubiquitous on plant surfaces, potentially dominating the phyllosphere population (Corpe and Rheem 1989). In this study, a pink-pigmented, aerobic, facultatively methylotrophic bacterial strain CBMB27^T isolated from leaf of rice (*Oryza sativa* L. cv Dong-Jin) was analyzed by a polyphasic taxonomic study.

Methods

Medium used for isolation

Strains CBMB27^T was isolated from surface-disinfected leaf tissues of rice (*Oryza sativa* L. 'Dong-Jin'). The strains were recovered on ammonium/mineral salts (AMS) medium (Whittenbury *et al.* 1970) supplemented with filter-sterilized cycloheximide (10 mg ml^{-1}) and methanol (0.5% v/v) at 28 °C. The strains were maintained routinely on nutrient agar (NA; Difco) medium, supplemented with 1% (v/v) methanol, or on selective AMS medium.

Scanning electron microscope (SEM)

Scanning electron microscope (SEM) observations were performed on fixed material that was prepared for routine examinations as described by Bozzola and Russell (1998). Samples were critical-point-dried, mounted on stubs, sputter-coated with gold/palladium and visualized by using a Hitachi S-2500C SEM with GEMINI column equipped with a field-emission electron source.

16S rRNA genes

16S rRNA genes were amplified using universal primers: fD1 and rP2 (Weisburg *et al.* 1991) and 16S rDNA sequencing was performed by big-dye primer method using an automated DNA sequencer (ABI Prism 310 Genetic Analyzer, Tokyo, Japan).

Nutritional features
Nutritional features were determined using the Biolog Microstation (MicroLog-3, 4.01B). The analysis was carried out in Biolog GN2 microtitre plates according to the manufacturer's instructions; the reactions were observed after incubating the plates at 28 °C for 7 days.

Carbon-source utilization tests
Carbon-source utilization tests (excluding biolog) were performed by using a standard protocol described by Green and Bousfield (1982).

Physiological and biochemical characteristics
Other physiological and biochemical characteristics were tested using the API ZYM and API 20NE galleries (bioMe´rieux) following the manufacturer's instructions. Cellular fatty acids were analysed in organisms grown on NA with 1% methanol (v/v) for 48 h.

Cellular fatty acids
The cellular fatty acids were derivatized to methylesters (Sasser 1990) and analyzed by a Gas Chromatograph (Hewlett Packard 6890) using Microbial Identification System (MIDI; Microbial ID) software package.

G+C content
The G+C content of genomic DNA was determined by HPLC analysis using a reverse-phase column (Supelcosil LC-18-S, Supelco) of individual nucleosides, resulting from DNA hydrolysis and dephosphorylation (Mesbah *et al*. 1989).

DNA-DNA hybridization
DNA-DNA hybridization was carried out following the filter hybridization method as described by Seldin and Dubnau (1985).

Results

Cells of strain CBMB27^T were Gram-negative, aerobic, non-endospore forming rods, frequently branched and occurring singly or in rosettes on solid AMS medium and formed pink to red-pigmented colonies. Photomicrograph of strain CBMB27^T grown on solid surface of AMS medium supplemented with 0.5% methanol is shown (Figure 1).

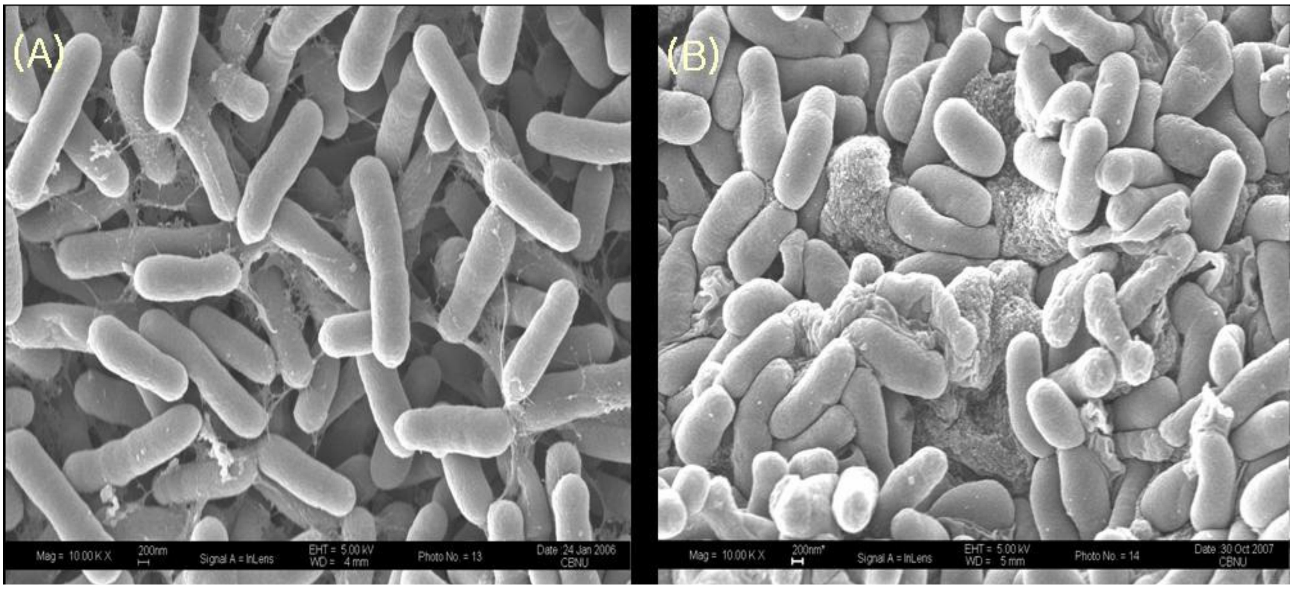

Figure 1. Scanning electron microscope (SEM) photomicrographs of *Methylobacterium phyllosphaerae* CBMB27^T on AMS medium (a) supplemented with 0.5% methanol (v/v) or NA medium (b) supplemented with 1% methanol (glutaraldehyde/osmium tetroxide fixation, gold/palladium coating: Hitachi S-2500C). Bar, 1µm and 200nm.

In this study, a pink-pigmented, aerobic, facultatively methylotrophic bacterial strain CBMB27^{T} isolated from leaf of rice (*Oryza sativa* L. cv Dong-Jin) was analyzed by a polyphasic taxonomic study. A comparative 16S rRNA gene sequence-based phylogenetic analysis placed the strain in a clade with the species *Methylobacterium oryzae*, *Methylobacterium fujisawaense*, and *Methylobacterium mesophilicum,* with which it showed sequence similarity of 98.3, 98.5, and 97.3%, respectively (Figure 2).

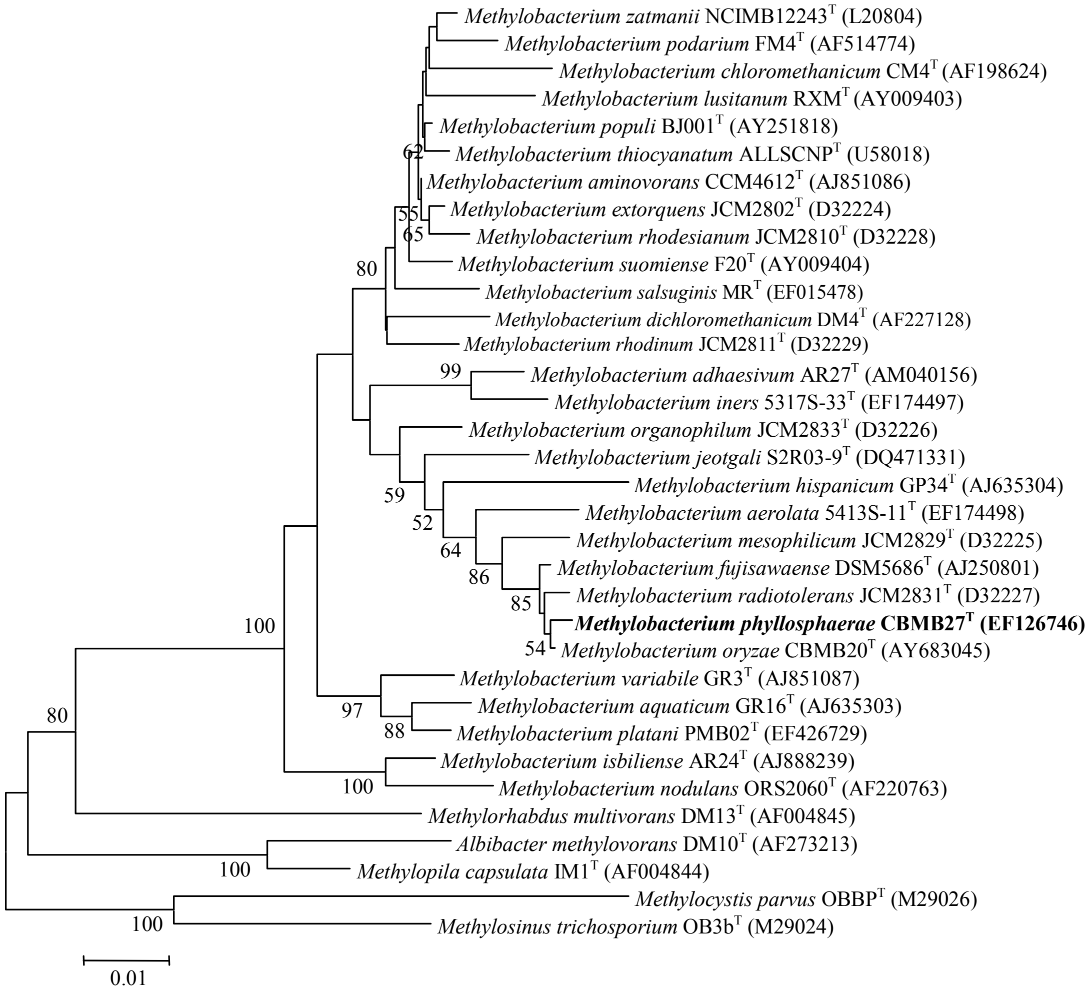

Figure 2. Phylogenetic tree based on 16S rRNA gene sequence comparison showing the position of the strain CBMB27^{T} and other related species of the genus *Methylobacterium*. The numbers at the nodes indicate the levels of the bootstrap support based on a neighbor-joining analysis of 1,000 resampled data sets. The bootstrap values below 50% were not indicated. Bar, 0.01 substitution per site.

However the DNA-DNA hybridization experiments revealed a low level (< 38%) of DNA-DNA relatedness for the strain CBMB27^{T} with its closest relatives. The major fatty acid was $C_{18:1}$ ω7c (Table 1) and the G+C content of the genomic DNA was 66.8mol%.

The type strain, CBMB27^{T} (LMG 24361 ^{T} = KACC 11716 ^{T} = DSM 19779 ^{T}), was isolated from leaf surface of rice (*Oryza sativa* L. cv Dong-Jin). Cellular fatty acids are: *cis*-7-octadecenoate (*cis* vaccenic acid, $C_{18:1}$ ω7c), 75.1%; major hydroxy fatty acids, 11.5%; hexadecanoate (palmitic acid, $C_{16:0}$), 4.1%; octadecanoate (stearic acid, $C_{18:0}$), 4.4% and 3-hydroxy octadecanoate ($C_{18:0}$ 3-OH), 0.9%.

Based on the results of 16S rRNA gene sequence analysis, DNA-DNA hybridization and physiological and biochemical characterization, that differentiated strain CBMB27^{T} from all recognized species of the genus *Methylobacterium*, it is concluded that strain CBMB27^{T} represents a new species in the genus *Methylobacterium* for which the name *Methylobacterium phyllosphaerae* sp. nov. is proposed (type strain CBMB27^{T} = LMG 24361^{T} = KACC 11716^{T} = DSM 19779^{T}).

Table 1. Cellular fatty acid compositions (as percentages of the total) of strain CBMB27^T and related species of the genus *Methylobacterium*
Species/strains: 1. CBMB27^T; 2. *M. oryzae* CBMB20 ^{T}; 3. *M. fujisawaense* KACC10744 ^{T}; 4. *M. mesophilicum* DSM 1708 ^{T}; 5. *M. radiotolerans* DSM 1819 ^{T}; 6. *M. extorquens* DSM 1337 ^{T}; 7, *M. adhaesivum* KACC 12195 ^{T}; 8, *M. hispanicum* DSM 16372 ^{T}; 9. *M. organophilum* DSM 760 ^{T}; 10. *M. iners* KACC11765 ^{T}; 11. *M. aerolatum* KACC11766 ^{T}; 12. *M. adhaesivum* KACC12195 ^{T}; 13. *M. platani* KCTC12901 ^{T}.
Values are percentages of total fatty acids; -, not detected. ECL, Equivalent chain-length. Fatty acids representing less than 0.3% in all strains were omitted.

Fatty acid	1	2	3	4	5	6	7	8	9	10	11	12	13
$C_{9:0}$	–	–	–	–	–	–	–	1.2	–	–	–	–	–
$C_{12:0}$	–	–	–	–	–	–	–	4.5	–	0.8	–	–	–
$C_{14:0}$	–	–	–	–	–	–	–	2.4	–	0.8	–	–	0.4
$C_{16:0}$	4.1	3.0	2.0	3.2	3.0	2.6	4.5	4.6	3.0	3.3	3.9	2.2	7.6
$C_{17:0}$ ISO 3-OH	11.5	–	–	–	–	9.1	–	–	–	–	–	–	–
$C_{18:0}$	4.4	4.6	5.4	4.1	5.3	2.3	–	4.6	6.3	2.9	2.4	1.1	1.7
$C_{18:0}$ 3-OH	0.9	0.8	0.7	1.5	0.6	–	–	–	0.5	–	1.7	2.0	3.5
$C_{18:1}$ ω7c	75.1	88.2	88.7	88.6	88.8	64.8	79.3	68.5	88.1	82.3	82.6	77.9	81.0
Summed Feature 2*	2.7	0.8	0.8	1.1	0.8	6.3	2.2	8.0	0.8	2.2	3.5	4.3	2.0
Summed Feature 3*	1.1	1.8	1.7	0.8	0.8	12.6	8.5	6.3	0.7	6.5	6.0	11.7	2.2
Summed Feature 4*	–	–	–	–	–	1.31	5.1	–	–	–	–	–	–
Unknown fatty acid 14.959 (ECL)	–	0.4	0.5	0.5	0.5	–	–	–	0.5	–	–	0.9	–

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 2 contained iso-6 $C_{16:1}$ I and/or $C_{14:0}$ 3-OH; Summed feature 3 contained $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH; Summed feature 4 contained iso-$C_{17:1}$ I and/or anteiso-$C_{17:0}$ B.

Conclusion

The 16S rRNA sequence similarity data, DNA-DNA hybridization values, and other phenotypic characteristics allowed the strain CBMB27^T to separate from other members of the genus *Methylobacterium.* On the basis of these results, strain CBMB27^T is considered to be a novel species of *Methylobacterium*, for which the name *Methylobacterium phyllosphaerae* sp. nov. is proposed.

References

Bozzola JJ, Russell LD (1998) 'Electron Microscopy', 2nd edn. (Sudbury, MA: Jones and Bartlett).

Corpe WA, Rheem S (1989) Ecology of the methylotrophic bacteria on living leaf surfaces. *FEMS Microbiol. Ecol.* **62,** 243–250.

Green PN (1992) The genus *Methylobacterium*. In 'The Prokaryotes', 2nd edn,. (Eds A Balows, HG Trüper, M Dworkin, W Harder, KH Schleifer). pp. 2342–2349 (New York: Springer).

Green PN, Bousfield IJ (1982). A taxonomic study of some Gram-negative facultatively methylotrophic bacteria. *J. Gen. Microbiol.* **128,** 623–638.

Mesbah M, Premachandran U, Whitman WB (1989) Precise measurement of the G+C content of deoxyribonucleic acid by highperformance liquid chromatography. *Int. J. Syst. Bacteriol.* **39,** 159–167.

Patt TE, Cole GC, Hanson RS (1976) Methylobacterium, a new genus of facultatively methylotrophic bacteria. *Int. J. Syst. Bacteriol.* **26,** 226–229.

Sasser M (1990) Identification of bacteria through fatty acid analysis. In 'Methods in Phytobacteriology'. (Eds S Klement, K Rudolf & D Sands) pp. 199–204. (Budapest Akademiai Kiado).

Seldin L, Dubnau D (1985) Deoxyribonucleic acid homology among *Bacillus polymyxa, Bacillus macerans, Bacillus azotofixans,* and other nitrogen-fixing Bacillus strains. *Int. J. Syst. Bacteriol.* **35,** 151–154.

Weisberg WG, Barns SM, Pelletier BA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173,** 697–703.

Whittenbury R, Phillips KC, Wilkinson JF (1970) Enrichment, isolation and some properties of methane-utilizing bacteria. *J. Gen. Microbiol.* **61,** 205–218.